Assessment of Central Retinal Function after Autologous Bone Marrow Derived Intravitreal Stem Cells Injection in Patients with Retinitis Pigmentosa using Multifocal ERG: A Pilot Study

Atul Kumar, SN Mohan Raj, Thirumalesh Basavaraj Mochi, Sujata Mohanty, Tulika Seth, Rajvardhan Azad

ABSTRACT

Purpose: To assess central retinal function after intravitreal bone marrow derived stem cells injection in patients with retinitis pigmentosa (RP) using multifocal (mf) ERG.

Study design: Prospective nonrandomized interventional study.

Materials and methods: Patients with RP with visual acuity (VA) ≤1.90 were included. All patients underwent mf-ERG testing (61 hexagons) prior to intravitreal stem cells injection. MF-ERG was repeated at end of 1st month, 3rd month and 6th month postinjection. First order kernel mf-ERGs were analyzed (amplitude and implicit time of n1 and p1).

Results: Thirty patients (26 male and 4 female) aged 18 to 58 years (mean 35.9) were included in the study. Visual acuity preinjection ranged from 0.48 to 1.9 logMAR (mean 1.25289 ± 0.5324). At 6 months of follow-up there was no statistically significant change in the best corrected visual acuity after stem cells injection (p = 0.785 Friedman test). At 6 months follow-up period mf-ERG p1 wave amplitude within 2º from fovea (ring 1) showed improvement (p-value 0.014). The p1 wave latencies also showed reduction in the implicit times (p-value 0.03). The maximum mean value of p1 wave amplitude was observed at 3 months of injection. The increase in P1 wave amplitude was maximal in ring 1. The change observed was statistically significant in ring 1 (p-value 0.014).

Conclusion: This study shows that autologous bone marrow stem cells transplantation by intravitreal injection is a safe procedure. Autologous stem cell injection improves and stabilizes central retinal visual function as shown by mf-ERG. Autologous bone marrow derived intravitreal stem cells injection may be a promising therapy in patients with retinitis pigmentosa and allied heredoretinal dystrophies, however larger sample size and longer follow-up period will be required to determine the long-term functional outcomes of such a therapy for these visually debilitating disorders.

Keywords: Retinitis pigmentosa, Stem cells, mf-ERG.

INTRODUCTION

Retinitis pigmentosa (RP) is a generic name for a group of hereditary disorders characterized by night-blindness, impaired dark adaptation, and a progressive visual field loss, which often leads to blindness. It is a diffuse retinal dystrophy with a prevalence of 1:4000.¹ It is a heritable group of disorder in which initial involvement of photoreceptors leads to subsequent damage to inner retinal cells.² Earliest visual symptom impairment manifests as nyctalopia,³ followed by a gradual reduction in peripheral vision. The long-term prognosis of patients with retinitis pigmentosa is poor, with eventual loss of central vision. Currently no definitive treatment for retinitis pigmentosa exists. The full-field electroretinogram (ERG) in RP typically shows a marked reduction of both rod and cone signals (extinguished in many cases), although rod loss generally predominates; a and b waves are reduced since the primary site of disease is at the photoreceptors and the retinal pigment epithelium (RPE). The ERG is usually abnormal in infancy or early childhood, except for some of the very mild and regional forms of RP. Recently, the multifocal electroretinogram (mf-ERG) has proven to be a valuable diagnostic aid. The mf-ERG technique developed by Sutter and Tran⁴ permits a topographical measurement of retinal response, thus providing an objective assessment of the central retinal function. The origin of retinal responses has been correlated to separate retinal layers and can indicate point of cellular dysfunction. The cone mediated mf-ERG with localized retinal stimulation may allow quantification of the remaining cone mediated function despite an advance stage of disease.⁵⁻¹⁰

MATERIALS AND METHODS

Subjects

This study was approved by the Ethics Committee, AIIMS. A written consent was taken before the surgery. Inclusion criterias were diagnosis of RP or allied heredoretinal dystrophies with best corrected VA 0.48 to 1.9 logMAR with media clarity, pupillary dilatation and patient cooperation sufficient for examination. Exclusion criterias were optic disk disease like glaucoma, optic atrophy from other causes, macular edema, age-related macular degeneration, bleeding disorders.

The period of study was November 2009 to November 2011.

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Conflict of interest: None declared
Written informed consent was taken from every patient and also the attendants of the patient in Hindi as well as in English and the consent form and format are approved by the Institute Ethic Committee (IEC-AIIMS) and Institute Committee for Stem Cell Therapy and Research (ICSCRT). The eye with poorer vision of the patient who fulfilled the inclusion criteria was selected for injection of stem cells and the fellow eye was taken as control. The decision was taken by the principle investigator—Professor Dr Atul Kumar.

VISUAL FUNCTION ASSESSMENT

Best corrected visual acuity was assessed on a logMAR (logarithm of the minimum angle of resolution) scale by using backlit Early Treatment Diabetic Retinopathy Study chart.

MULTIFOCAL (MF) ERG

The mf-ERG was recorded on metrovision system (France). Before recording from index eye, pupil was dilated with combination of 1% tropicamide and 5% phenylephrine topical eye drops. The cornea was anesthetized with 1% proparacaine before putting bipolar Burian-Allen contact lens electrode with a built-in infrared illuminator (Hansen Ophthalmic Development Laboratory, Coralville, IA and EDI) was placed on the corneal surface of the eye according to instructions in the full-field ERG or pattern ERG standards of the International Society for Clinical Electrophysiology and Vision (ISCEV). Refractive correction was given by +3D spherical lenses in front of corneal electrode. The stimulus consisted of 61 regular hexagons with viewing distance at 33 cm. which corresponds to field of 30º horizontally and 24º vertically flashed in a pseudorandom pattern on a dark background cover with a luminance set at 30 cd/m². The standard stimulation based on black/white with blue background to minimize rod response and maximize cone responses. The stimulus frequency was set at 17 Hz to optimize the amplitude of responses. The fellow eye was occluded with an opaque black patch. The total duration of the mf-ERG per eye was 5 minutes. The protocol followed the recommended guidelines of the International Society for Electrophysiology of Vision (ISCEV) for basic mf-ERG.11,12

PROCESSING OF BONE MARROW

All open cell handling procedures were performed in class 100 environments. Harvested bone marrow sample were diluted 1:3 with phosphate buffer saline (PBS). Mononuclear cells were separated by Ficoll density separation method. The diluted sample were layered over Ficoll medium (Specific gravity 1.077) in 50 ml tubes. Ficoll was warmed to room temperature before use. Sample was centrifuged at a speed of 700 G for 25 minutes at 25°C. After centrifugation interface cells which form the whitish ring were aspirated out into a separate tube. Rest of the product was kept aside in sterile container till the final product is released. Cell suspension was washed with sterile PBS to remove traces of Ficoll by centrifuging it thrice at 400 G for 5 minutes at 25°C. Supernatant of each wash was kept in sterile container till final product a cell pellet was re-suspended in 1 ml syringe. The final cell concentration was 8 million mononuclear cells per 0.1 ml (Fig. 1).

Mononuclear Cells Evaluation

An aliquot of harvested mononuclear cells were evaluated for:

- Viability: Trypan blue dye exclusion test was done to know the percentage of live cells. Cell viability of more than 90% was acceptable.

Bone Marrow (BM) Aspiration

The patient was placed in the lateral decubitus position, with the top leg flexed and the lower leg straight. The site was prepared, cleaned with an antiseptic (Betadine) scrub, and draped, exposing the iliac crest. The skin and the area down to the periosteuam was infiltrated with a local anesthetic (approximately 10 cc of 1% xylocaine was used). The BM aspiration needle, with a stylet in place, was inserted, advanced by rotating clockwise and counterclockwise slowly until the cortical bone was penetrated and the marrow cavity was entered, once within the marrow cavity, the stylet was removed, and using a 20 cc syringe, approximately 25 to 35 cc of BM was aspirated.
• Cell morphology assessed using Giemsa stain.
• **Total cell count:** Cell number was assessed by counting in the Neubaur chamber under microscope.
• **Characterization:** The bone marrow mononuclear cells were characterized using a panel of antibodies (CD-34, CD-45, CD-3, CD-4, and CD-8) by flow cytometry.

**Sterility**

An aliquote of bone marrow and isolated mononuclear cells was sent for microbiological culture evaluation. The results of microbial cultures were reviewed by the laboratory in charge or designee in a timely manner. No growth of microorganisms was noted in any of the culture in our study.

**FLOW CYTOMETRY**

Around $0.5 \times 10^6$ MNCs from bone marrow were stained with CD-34, CD-3 (BD PharMingen), CD-4 (BD PharMingen) and CD-8 (BD PharMingen) for 30 minutes at 4ºC. Parallel appropriate isotope controls were also stained. All samples were rinsed twice in PBS and analyzed on a FACS LSR-II (BD Biosciences) and analyzed using software FACS DIVA 6.12 (BD Biosciences). At least 10,000 cells in total were analyzed (Figs 2 and 3).

**RESULTS**

The mean age of patients was 33.7 years (range 19 to 60 years). The mean volume of bone marrow aspirated was 34 ± 8 ml. The total cell count was 8 million per ml with viability being 99% ± 1%. The mean final volume was around 4.5 ± 0.5 ml with a mean CD 34 and CD 45 count of 0.163 ± 0.17, a mean CD-3 count of 11.6 ± 5.7, and a mean CD4 count of 5.39 ± 3.3, a mean CD-8 count of 6.5 ± 6.2.

**INTRAVITREAL INJECTION**

**Transplantation of BM-MNC:** MNC were suspended in physiological saline and 4 to 5 million cells suspended in 0.1 ml of saline solution were injected into the mid vitreous with the help of 26G needle by the pars plana route under topical anesthesia.

Paracentesis was done to avoid sudden peak in the IOP following intravitreal injection as the volume of stem cells was 0.15 ml and is sufficient to cause acute IOP rise.

Any sign of infection like endophthalmitis, intraocular pressure elevation, vitreous hemorrhage, cataract development or progression and anterior chamber reaction was noted on subsequent follow-ups.

**RESULTS**

Thirty patients (26 male and 4 female) aged 18 to 58 years (mean 35.9) were included in the study. Two had Usher’s syndrome and seven patients had positive family history. The eye in which intravitreal stem cells were injected was considered as the study eye and the fellow eye of the same patient was included as the control eye.

Visual acuity preinjection ranged from 0.48 to 1.9 logMAR (mean 1.25289 ± 0.5324). At 6 months of follow-up there
Fig. 2: Representative plot for CD3, CD4, CD8 enumeration by flow cytometry

Fig. 3: Representative plot for CD34 enumeration. Quantification of MNCs by flow cytometry. Cell distribution based on FSC (forward scatters) and SSC (side scatter) parameter that describe their size and granularity representatively and ring analysis charts

was no statistically significant change in the best corrected visual acuity after stem cells injection (p = 0.785 Friedman Test) as shown in Table 1.

Fundus photographs of the patients did not reveal any noticeable changes over 6 months follow-up in both treated and control group.

MULTIFOCAL ERG

There was increase in p1 wave amplitude in ring 1 and ring 2 in 6 months follow-up period. In the study eye p1 wave amplitude at 2 to 5º from fovea showed improvement, improvement being statistically significant (p: 0.014) as shown in Table 2. The latency of p1 wave between 5 to 10º showed statistically significant improvement (p: 0.030) as shown in Table 3. No such improvements were noted in the control eyes as shown in Tables 4 and 5. The improvement was seen to be stable at 6 months follow-up as shown in Figure 1 and also in Tables 2, 3 and Figures 4 to 11.

DISCUSSION

The use of autologous bone marrow derived stem cells for retinal degenerations like retinitis pigmentosa offer
Assessment of Central Retinal Function after Autologous Bone Marrow Derived Intravitreal Stem Cells Injection

TABLE 1: Best corrected visual acuity—cases and controls

<table>
<thead>
<tr>
<th></th>
<th>Preoperative</th>
<th>1st month</th>
<th>3rd month</th>
<th>6th month</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study eye</td>
<td>1.2529 ± 0.5324</td>
<td>1.2468 ± 0.5292</td>
<td>1.2276 ± 0.5347</td>
<td>1.2277 ± 0.5289</td>
<td>0.785</td>
</tr>
<tr>
<td>Control eye</td>
<td>1.1614 ± 0.5103</td>
<td>1.1659 ± 0.5292</td>
<td>1.1660 ± 0.5293</td>
<td>1.1659 ± 0.5292</td>
<td>0.709</td>
</tr>
</tbody>
</table>

TABLE 2: Multifocal ERG p1 amplitude—study eyes

<table>
<thead>
<tr>
<th></th>
<th>Ring 1 (&lt;2º)</th>
<th>Ring 2 (2-5º)</th>
<th>Ring 3 (5-10º)</th>
<th>Ring 4 (10-15º)</th>
<th>Ring 5 (&gt;15º)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preoperative</td>
<td>223.29 ± 227.29</td>
<td>105.14 ± 121.23</td>
<td>211.59 ± 234.91</td>
<td>179.48 ± 220.19</td>
<td>158.27 ± 210.12</td>
</tr>
<tr>
<td>1st month</td>
<td>238.58 ± 222.17</td>
<td>82.05 ± 99.97</td>
<td>257.79 ± 329.44</td>
<td>260.55 ± 280.16</td>
<td>165.51 ± 186.12</td>
</tr>
<tr>
<td>3rd month</td>
<td>279.91 ± 210.6</td>
<td>160.96 ± 148.9</td>
<td>267.08 ± 252.38</td>
<td>225.27 ± 213.01</td>
<td>172.65 ± 112.08</td>
</tr>
<tr>
<td>6th month</td>
<td>258.46 ± 177.03</td>
<td>146.83 ± 148.64</td>
<td>242.93 ± 174.63</td>
<td>198.19 ± 169.74</td>
<td>180.30 ± 148.12</td>
</tr>
</tbody>
</table>

TABLE 3: Multifocal ERG p1 amplitude—control eyes

<table>
<thead>
<tr>
<th></th>
<th>Ring 1 (&lt;2º)</th>
<th>Ring 2 (2-5º)</th>
<th>Ring 3 (5-10º)</th>
<th>Ring 4 (10-15º)</th>
<th>Ring 5 (&gt;15º)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preoperative</td>
<td>269.91 ± 287.32</td>
<td>90.04 ± 124.14</td>
<td>215.02 ± 255.64</td>
<td>180.32 ± 232.7</td>
<td>203.38 ± 144.67</td>
</tr>
<tr>
<td>1st month</td>
<td>266.13 ± 293.94</td>
<td>87.36 ± 110.24</td>
<td>228.7 ± 248.98</td>
<td>244.73 ± 271.52</td>
<td>227.26 ± 149.51</td>
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<tr>
<td>3rd month</td>
<td>241.47 ± 337.27</td>
<td>125.92 ± 151.89</td>
<td>226.8 ± 276.73</td>
<td>237.06 ± 210.89</td>
<td>234.57 ± 154.9</td>
</tr>
<tr>
<td>6th month</td>
<td>200.93 ± 271.28</td>
<td>101.9 ± 152.49</td>
<td>212.84 ± 192.00</td>
<td>143.74 ± 144.837</td>
<td>213.69 ± 104.35</td>
</tr>
</tbody>
</table>

TABLE 4: Multifocal ERG p1 implicit time—study eyes

<table>
<thead>
<tr>
<th></th>
<th>Ring 1 (&lt;2º)</th>
<th>Ring 2 (2-5º)</th>
<th>Ring 3 (5-10º)</th>
<th>Ring 4 (10-15º)</th>
<th>Ring 5 (&gt;15º)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preoperative</td>
<td>29.00 ± 15.65</td>
<td>48.04 ± 23.31</td>
<td>28.88 ± 18.34</td>
<td>29.07 ± 25.24</td>
<td>32.67 ± 18.49</td>
</tr>
<tr>
<td>1st month</td>
<td>22.02 ± 16.15</td>
<td>52.11 ± 20.52</td>
<td>20.58 ± 22.90</td>
<td>32.02 ± 21.98</td>
<td>26.21 ± 26.63</td>
</tr>
<tr>
<td>3rd month</td>
<td>26.55 ± 20.09</td>
<td>42.68 ± 23.05</td>
<td>21.99 ± 25.35</td>
<td>26.55 ± 15.12</td>
<td>31.44 ± 30.00</td>
</tr>
<tr>
<td>6th month</td>
<td>27.13 ± 21.97</td>
<td>42.91 ± 24.10</td>
<td>19.08 ± 26.31</td>
<td>27.13 ± 22.04</td>
<td>30.79 ± 25.78</td>
</tr>
</tbody>
</table>

TABLE 5: Multifocal ERG p1 implicit time—control eyes

<table>
<thead>
<tr>
<th></th>
<th>Ring 1 (&lt;2º)</th>
<th>Ring 2 (2-5º)</th>
<th>Ring 3 (5-10º)</th>
<th>Ring 4 (10-15º)</th>
<th>Ring 5 (&gt;15º)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preoperative</td>
<td>28.34 ± 19.01</td>
<td>28.88 ± 23.04</td>
<td>22.47 ± 17.64</td>
<td>28.34 ± 25.06</td>
<td>21.68 ± 17.34</td>
</tr>
<tr>
<td>1st month</td>
<td>30.58 ± 17.23</td>
<td>39.40 ± 21.57</td>
<td>25.90 ± 23.63</td>
<td>30.4 ± 21.35</td>
<td>17.68 ± 17.42</td>
</tr>
<tr>
<td>3rd month</td>
<td>31.99 ± 32.32</td>
<td>32.45 ± 25.27</td>
<td>23.27 ± 28.12</td>
<td>29.22 ± 13.75</td>
<td>19.71 ± 16.23</td>
</tr>
<tr>
<td>6th month</td>
<td>33.08 ± 33.4</td>
<td>33.50 ± 26.24</td>
<td>28.84 ± 25.39</td>
<td>30.11 ± 24.2</td>
<td>20.99 ± 15.97</td>
</tr>
</tbody>
</table>

photoreceptors neuroprotection and rescue from degeneration without immune rejection and might be a promising modality to treat retinitis pigmentosa. Currently, there is no definitive treatment available for retinitis pigmentosa.

Stem cells are defined as cells that have clonogenic and self-renewing capabilities and that differentiate into multiple cell lineages (Weissman 2000). However, this classic paradigm of stem cell differentiation restricted to its organ-specific lineage is being challenged by the suggestion that adult stem cells, including hematopoietic stem cells, retain a previously unrecognized degree of developmental plasticity that allows them to differentiate across boundaries of lineage, tissue, and germ layer. The hierarchical view no longer seems correct.

In the past decade, there has been a better understanding of the bone marrow stem cell characteristics and differentiation potential. Bone marrow derived stem cells have been suggested to contribute to repair and genesis of cells specific for liver, cardiac and skeletal muscle, (Weissman 2000).
The mechanism involved has been termed transdifferentiation, although other explanations including cell fusion have been postulated. Using adult stem cells to generate or repair solid organ tissue obviates the immunologic, ethical, and teratogenic issues that accompany embryonic stem cells (Korbling 2003).

Our work was based on the prior observations of Otani and others which showed that by injecting autologous bone marrow stem cells intravitreally causes stabilization of the retinal vasculature and the stem cells integrate into and associate with degenerating vessels, and this can prevent loss of cones in two mouse models of retinal degeneration, rdl and rdl0. This effect was observed following delivery of bone marrow derived stem cells from genetically defective mice as well as bone marrow derived stem cells from wild-type mice.

This observation in mice suggests that in humans, the patient’s own bone marrow cells (even from patients with a genetic defect) might provide an efficacious neuroprotective and vasculotropic effect. This effect may preserve central
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vision and it also does not have any side effects associated with the use of viral vectors in long-term gene therapy. There is also no possibility of any kind of rejection. Thus, the use of autologous bone marrow stem cells remains an only alternative for these potentially incurable blinding diseases.

The intravitreally injected bone marrow stem cells help in preserving the degenerating retina in more than one way. As shown by Otani et al stem cells cause significant upregulation of many antiapoptotic genes, including small heat shock proteins and transcription factors in the two mouse models of retinal degeneration, rdl and rd10. Second, the injected stem cells may differentiate into retinal neural cells as shown by Tomita et al.

This is the first clinical study in world literature evaluating the intravitreal transplantation of bone marrow stem cells in retinal degenerations in humans.

Fig. 6: Patient 1: Multifocal ERG trace array 6 months poststem cell injection intravitreal injection of stem cells

Fig. 7: Patient 1: Multifocal ERG ring analysis 6 months poststem cell injection intravitreal injection of stem cells
In our study the trend of change of the studied parameters with time (at various follow-ups) was studied. At 6 months, follow-up period multifocal ERG p1 wave amplitude in central field (with in 2º from fovea) showed improvement (p-value <0.05). The maximum mean value of p1 wave amplitude was observed at 3 months. Three patients developed postoperative uveitis on day second after injection which was controlled with systemic steroid within a week. In remaining patients up to the minimum follow-up period of 6 months, no adverse effects like anterior chamber reaction, intraocular pressure elevation, vitreous inflammation or hemorrhage and cataract development or progression, epiretinal membrane, etc. were noted.

Otani et al\cite{19} showed in their work that stem cells effect observed at 2 months after intravitreal injection and for as long as 6 months. They had also showed increase survival of cones after stem cell injection. We found similar observation in our study. As shown in Table 2 (mean value of mf-ERG p1 amplitude) maximum mean values were observed at 3rd month in inner rings, i.e. 1, 2 and 3. However, change was significant only in ring 1 its further shows that stem cells rescue the photoreceptors form undergoing degeneration by exerting neurotrophic and neuroprotective effect.

The predominantly rescued cells in the stem cell injected eye were the cones in the mouse models rd1 and rd10 in the Otani and colleagues study. In our study in the multifocal ERG examination of the retinitis pigmentosa group, the p1 wave amplitude at 2 to 5º from fovea showed statistically significant improvement (p: 0.014) from the preinjection levels. This is the cone dominated area of the retina and correlates well with the histopathological examination of the mice retina in Otani’s experiment.

However, there was no improvement in visual acuity, but it remained stable throughout the period of follow-up. This study shows that stem cells might be useful in halting the process of degeneration in retinal degenerative disorders. However, The long-term efficacy and dose standardization is still to be determined. The improvement seen on mf-ERG found to be stable after 6 months suggesting that injections of stem cells may be helpful in prevention of photoreceptor degeneration and stabilization of the RPE and photoreceptors and may delay photoreceptor degeneration. The potential benefit of stem cells in retinal degenerative
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disorders gives a ray of hope for the patients suffering from these blinding diseases.

CONCLUSION

This study shows that autologous bone marrow stem cells transplantation by intravitreal injection is a safe procedure and may be a promising therapy in retinal degenerations like retinitis pigmentosa.

REFERENCES


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